2. Mercuric chloride and silver nitrate have a qualitatively similar inhibitory action on the enzyme. This appears primarily to be competitive, but an effect on K_s , the dissociation constant of the inactive ES_2 enzyme-substrate complex, can also be distinguished, thereby increasing substrate inhibition. A third effect develops only in the presence of substrate, increasing the overall inhibition. Phenylmercuric hydroxide exhibits a novel form of inhibition in that it only decreases K_s . *p*-Chloromercuribenzoate is a purely competitive inhibitor and appears not to affect K_s . Cupric sulphate probably inhibits competitively and also decreases K_s , so combining the two independent actions of the organic mercurials.

3. The inactivating action of copper is potentiated by various reducing agents. Ascorbate is shown to act by reducing cupric copper to the much more toxic cuprous form. In the presence of bisulphite, copper catalyses a substrate-protected decomposition of the enzyme.

4. It is concluded that both SH and SS groups are involved in the active centre of β -glucuronidase.

5. Cuprous ion is shown to cause a non-enzymic hydrolysis of several aryl glucosides, including phenolphthalein β -glucuronide.

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Polysaccharides of *Chlorella pyrenoidosa*

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Although the polysaccharides of the green algae (Chlorophyceae) have not been investigated as extensively as those of the red and brown algae, the few that have been studied seem to show certain structural characteristics and are different from those of the other classes of algae. In most of

the work on the water-soluble polysaccharides of the green algae criteria of homogeneity have not been established for the materials isolated and therefore it is difficult to assess the significance of the structural work carried out on these products. However, from the work on the polysaccharides

isolated from *Ulva lactuca* (Brading, Georg-Plant & Hardy, 1954), *Acrosiphonia centralis* (O'Donnel & Percival, 1959a), *Cladophora rupestris* (Fisher & Percival, 1957; O'Donnel & Percival, 1959b) and *Caulerpa filiformis* (Mackie & Percival, 1959, 1960) it is apparent that rhamnose commonly occurs and that the complex polysaccharides contain a wide variety of other sugar residues.

Northcote, Goulding & Horne (1958) have described the chemical composition and structure of the cell wall of *Chlorella pyrenoidosa*. A knowledge of the chemical nature of the polysaccharides in the plant cell wall is important for any biosynthetic studies on this complex organized structure. This paper describes the isolation, purification and structural studies on the alkali-soluble polysaccharides from the whole cells of this alga.

EXPERIMENTAL

Materials

Chlorella pyrenoidosa. This alga was grown under sterile conditions as described by Northcote *et al.* (1958). The culture, plated on casein hydrolysate, glucose, meat extract and agar medium, showed no bacterial contamination when incubated at 37° for 18 hr.

Enzyme preparations. Crude amylase was obtained from human saliva by rinsing out the mouth with 25 ml. of water at 25° and centrifuging the resultant solution. The clear supernatant was used. Cellulase was prepared from *Helix pomatia* (Myers & Northcote, 1958, 1959). β -Galactosidase was kindly given by Mr M. H. Procter and was prepared from *Bacillus megaterium* (Procter & Wilson, 1958).

Analytical methods

Measurements of pH. These were made with a Pye pH meter and a glass electrode at room temperature and by standardizing the apparatus with standard buffer tablets, pH 4.0 and 6.99 (Burrroughs Wellcome and Co.).

Evaporations. All evaporations were carried out under reduced pressure at temperatures below 50°.

Paper chromatography. Free sugars were run by descending chromatography on Whatman no. 1 papers with ethyl acetate-pyridine-water (2:1:2, by vol.) for 4 hr. (Jermyn & Isherwood, 1949). ethyl acetate-pyridine-water (8:2:1, by vol.) for 16–18 hr. Methylated sugars were run on Whatman no. 1 paper either with butan-1-ol saturated with water for 12 hr. or ethyl methyl ketone saturated with water for 12 hr. (Boggs, Cuendet, Ehrenthal, Koch & Smith, 1950).

Sugar spots were located with aniline hydrogen phthalate (Partridge, 1949), and in some cases, for the detection of oligosaccharides and trace amounts of sugars, alkaline AgNO_3 reagent (Trevelyan, Procter & Harrison, 1950) was used.

For quantitative work 5–20 μl . of the sugar solutions was applied to the chromatograms and after separation the individual sugars were estimated by two methods: (i) elution of the sugars with water and colorimetric estimation of the solution after reaction with *o*-aminobiphenyl according to the method of Timell, Glaudemans & Currie (1956); (ii) elution of the aniline phthalate-coloured spots with

0.1N-HCl in aq. 80% (v/v) ethanol and subsequent colorimetric estimations of the solution according to the method of Wilson (1959).

Preparative column chromatography. A column (9 cm. diam.) containing a mixture of activated charcoal (250 g.; British Drug Houses Ltd.) and Celite no. 545 (250 g.; Johns Manville Co., New York, U.S.A.) was prepared (Whistler & Durso, 1950; Whelan, Bailey & Roberts, 1953) and oligosaccharides were eluted from the column by means of aq. 2.5–30% (v/v) ethanol. Fractions (1 l.) were collected.

A cellulose column (30 cm. $\times$ 4 cm.) was used for the separation of monosaccharide mixtures (Hough, Jones & Wadman, 1949). The column was run with propan-2-ol-water (9:1) and an initial flow rate of 25 ml./hr., which was reduced to 15 ml./hr. when mannose and arabinose were eluted. Fractions (5 ml.) were collected and the sugars present in each were identified by paper chromatography. The fractions were combined, filtered and concentrated.

Electrophoresis of polysaccharides. This was done on glass-fibre paper by the method of Fuller & Northcote (1956). A current (800 v, 40 ma) was passed for 1 hr. and the paper was dried at room temperature.

Solutions of polysaccharides (1%) were examined in borate buffer in a Tiselius apparatus (Perkin-Elmer and Co., U.S.A.; model 38) according to the method described by Northcote (1954). The experiments lasted about 4–5 hr. and pictures were taken at 40 min. intervals.

Hydrolysis of sugars and polysaccharides. Samples were hydrolysed completely with an equal weight of Amberlite resin IR-120 (H) and a trace of 2N- H_2SO_4 in sealed tubes at 100° for 12 hr. (Wadman, 1952). The recovery of each sugar after the hydrolysis and subsequent quantitative chromatographic procedure was determined (Table 1).

The polysaccharides were partially hydrolysed by heating solutions (1%, w/v) in 0.33N- H_2SO_4 at 100° for 165 min. The hydrolysates were neutralized with BaCO_3 , concentrated and analysed by chromatography on paper and on charcoal and cellulose columns.

Sedimentation measurements. Solutions (0.3–2%) in 0.1M- KH_2PO_4 were sedimented at $2.69 \times 10^5 g$ for about 3 hr. in the Spinco analytical ultracentrifuge, model E (Special Instruments Co., Belmont, Calif., U.S.A.). Pictures were taken every 16 or 32 min. Sedimentation coefficients were corrected to 20°.

Diffusion measurements. These were made over a period of 3 days on solutions of polysaccharides (1 and 2%) in 0.1M- KH_2PO_4 (pH 5.3). The Tiselius electrophoresis

Table 1. *Percentage recovery of sugars after hydrolysis*

Hydrolysis of each sugar was done with Amberlite resin IR-120 (H) and a trace of 2N- H_2SO_4 for 12 hr. and was followed by quantitative chromatography (Wadman, 1952; Wilson, 1959). Results are given $\pm$ s.d.; 35 experiments were done for each sugar.

Sugar	Percentage recovery
Galactose	72.1 $\pm$ 3.4
Glucose	71.8 $\pm$ 3.7
Mannose	72.0 $\pm$ 3.8
Arabinose	68.7 $\pm$ 4.2
Xylose	69.2 $\pm$ 3.4
Rhamnose	69.4 $\pm$ 3.1

apparatus was used. The diffusion coefficients were corrected to 20°.

Partial specific volume. This was determined for the polysaccharide at 20° in a pycnometer (vol. 2.7 ml.).

Viscosity measurements. The determinations were performed in aqueous solutions (0.25–2%) in a horizontal capillary viscometer (Tsuda, 1928; Ostwald, 1933). The capillary had the following characteristics: radius 0.0385 cm.; length 25.50 cm.; flow time 10 sec. for water, 10 cm. length at a pressure of 63.0 cm. water. Experiments were carried out at 23.5°.

Elementary analysis. All the analyses were carried out on material dried at 0.01 mm. Hg over P_2O_5 at room temperature.

Total N was determined by micro-Kjeldahl digestion

(Chibnall, Rees & Williams, 1943), followed by distillation and titration.

Total P was determined according to the method of Fiske & Subbarow (1925).

Ash content was determined by maintaining a dried sample at red heat (approx. 800°) in a platinum boat in a stream of dry air for 1–2 hr.

RESULTS

Isolation of the alkali-soluble polysaccharides

The alkali-soluble polysaccharides were isolated from freeze-dried whole cells by the method indicated in Fig. 1. This is based on the procedure described by Northcote *et al.*

Fig. 1. Isolation of the polysaccharides from *Chlorella*.

(1958). The yields at various stages are shown and they represent average values for ten extractions. The alkali-soluble material obtained (Fig. 1) was identical with that described by Northcote *et al.* (1958). It contained both hemicellulose and starch, and on electrophoresis in the Tiselius apparatus it gave two major components (Fig. 2a) with electrophoretic mobilities of $3.6 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$ and $9.0 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$. These could be separated on glass-fibre paper by electrophoresis; the slower-moving component stained blue with iodine and contained starch.

When the starch was removed from the crude material with salivary amylase (Northcote *et al.* 1958) the yield of hemicellulose was 69% for material obtained from *Chlorella* grown in autumn and winter, and 56% for that obtained from spring and summer cultures of the algae. The seasonal variation in starch content was related to the length of the day during which the *Chlorella* was grown. This purification was followed by examination of the material in the Tiselius apparatus after starch removal (Fig. 2b).

It was found that not all preparations of the alkali-soluble polysaccharides could be purified in this way. In an experiment in which 720 mg. of crude hemicellulose was repeatedly incubated with amylase seven times, under toluene, over a period of 3 weeks, the hemicellulose obtained was not starch-free when tested with iodine solution.

Fig. 2. (a) Boundary electrophoresis of the alkali-soluble polysaccharides (10 mg./ml.) in 0.05M-borate buffer, pH 9.2. Descending limb, 40 min. (b) Boundary electrophoresis of the alkali-soluble polysaccharide after treatment with salivary amylase (10 mg./ml.) in 0.05M-borate buffer, pH 9.2. Descending limb, 40 min.

Preparative column electrophoresis. The crude polysaccharide (150 mg.) in 2 ml. of borate buffer (pH 9.2, 0.05M) was applied to a glass-powder column (100 cm. $\times$ 2.5 cm.) (Hocevar & Northcote, 1957). Electrophoresis was maintained at 450 v, 30 mA for 27 hr., after which the fractions were eluted off the column with the buffer. Fractions (5 ml.) were collected and each was analysed for total hexoses by treatment of a sample (0.1 ml.) with anthrone reagent (Seifter, Dayton, Novic & Muntwyler, 1950), for

No. of tube containing fractions (5 ml.) collected from the column

Fig. 3. Electrophoresis of alkali-soluble polysaccharides (approx. 150 mg.) in 0.05M-borate buffer, pH 9.2, at 1.5 v/cm. for 27 hr. on a glass-powder column. Colorimetric estimation of each tube with (a) anthrone, (b) phloroglucinol, (c) iodine.

total pentoses by treatment of a sample (0.4 ml.) with phloroglucinol reagent (Dische & Borenfreund, 1957) and for total starch by treatment of a sample (0.1 ml.) with iodine reagent (McCready, Guggolz, Silveira & Owens, 1950; Nielsen & Gleason, 1945). A good separation of the starch and the hemicellulose (hemicellulose A) was obtained (Fig. 3*a, b, c*). The average recovery from the column was $90 \pm 2\%$ (50 experiments). In many experiments a partial separation of the starch fraction into amylose and amylopectin components was obtained. The purified fractions were each applied again to the column and electrophoresis was maintained under the same conditions as before. Only one peak was obtained in each case. The starch:hemicellulose A ratio was 1:3. Each fraction moved as a single component when examined electrophoretically on glass-

Fig. 4. Boundary electrophoresis of purified hemicellulose A (10 mg./ml.) in 0.05M-borate buffer, pH 9.2. Ascending limb, 40 min.

Fig. 5. Boundary electrophoresis of the starch fraction obtained from the glass-powder column, 10 mg./ml., in 0.05M-borate buffer, pH 9.2. (a) Unfractionated material (ascending limb, 40 min.). (b) Hemicellulose B isolated after removal of starch from a solution in borate buffer with cetyltrimethylammonium bromide (ascending limb, 40 min.). (c) Pure starch from which hemicellulose B has been removed (descending limb, 40 min.).

fibre paper and in the Tiselius apparatus (Figs. 4 and 5*a*). The electrophoretic mobilities were $10.30 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$ for hemicellulose A, and $3.75 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$ for the starch fraction.

Hemicellulose A could not be further fractionated from solution by the use of varying concentrations of $(\text{NH}_4)_2\text{SO}_4$, acetic acid or ethanol. Addition of cetyltrimethylammonium bromide to the borate complex of the polysaccharide yielded no new fractions. Hemicellulose A contained no N, P, SO_4^{2-} ions or ash; $[\alpha]_D^{18} + 11^\circ$ (c 1.0 in water; *l*, 1.0).

The starch component from the glass-powder column when hydrolysed yielded sugars other than glucose (Olaitan & Northcote, 1961) and the starch in the mixture could be completely digested by salivary amylase. The material was further fractionated with cetyltrimethylammonium bromide (Barker, Stacey & Zweifel, 1957). The material (542 mg.) was dissolved in 50 ml. of water and to the solution were successively added 50 ml. of 0.05M-borate buffer (pH 9.2), 50 ml. of 5% cetyltrimethylammonium bromide solution and 2 ml. of 0.5N-NaOH (Bouveng & Lindberg, 1958). The white precipitate obtained was dissolved in a minimal amount of 2N-acetic acid and reprecipitated in 400 ml. of ethanol saturated with NaI. The precipitate was collected, washed four times each with ethanol and ether and dried. The material was dissolved in water, dialysed for 48 hr. against running tap water and 24 hr. against distilled water to remove all adhering reagent and reprecipitated. The white product (144 mg.) gave a blue colour with iodine and on complete acid hydrolysis gave glucose only. This was pure *Chlorella* starch, $[\alpha]_D^{18} + 135.0^\circ$ (c 1.0 in water; *l*, 1.0). It contained no N or ash; glucose content was 97.8% when estimated with the anthrone reagent (Seifter *et al.* 1950).

The supernatant was refractionated twice with the cetyltrimethylammonium bromide and the combined supernatants were added to 700 ml. of ethanol saturated with NaI. A fine white precipitate was obtained. This was collected, washed with ethanol and ether and dried. The product was dissolved in water, dialysed and reprecipitated as described for the starch component. Yield 232 mg. This material was another polysaccharide (hemicellulose B) and was starch-free. $[\alpha]_D^{18} + 75.0^\circ$ (c, 1 in water; *l*, 1). It contained no N, P, SO_4^{2-} ions or ash. On acid hydrolysis it gave glucose (25.0 moles), galactose (13.0 moles), mannose (2.0 moles), arabinose (1.0 mole), xylose (1.0 mole), rhamnose (12.0 moles) and an unidentified sugar (20.0 moles) (Olaitan & Northcote, 1961).

The purified *Chlorella* starch and hemicellulose B each gave single peaks when investigated electrophoretically in a Tiselius apparatus (Figs. 5*b, c*). The electrophoretic mobility for *Chlorella* starch was $3.75 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$ and for hemicellulose B, $3.75 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$.

Hemicellulose A, hemicellulose B and *Chlorella* starch were found to be present in an approximate ratio of 9:2:1 respectively.

Physical properties of hemicellulose A

Molecular weight. The sedimentation diagram showed one symmetrical peak (Fig. 6). The dependence of the sedimentation coefficient (S_{20}) on the concentration of the polysaccharide is shown in Fig. 7. S_{20} for a 1% solution was found to be $2.65 \times 10^{-13} \text{ cm. g}^{-1} \text{ sec.}^{-1}$. The partial specific volume $\bar{V}$ was 0.65 ml./g. and the diffusion coefficient, D_{20} , was $6.8 \times 10^{-7} \text{ cm.}^2 \text{ sec.}^{-1}$.

Fig. 6. Sedimentation pattern of pure hemicellulose A dissolved in 0.1M-KH₂PO₄, pH 5.3 (15 mg./ml.). Centrifugal force was $2.69 \times 10^5 g$; temp. 21°. Photographs were taken at 16 min. intervals. Migration is from right to left.

Fig. 7. Variation of S_{20} with concentration of hemicellulose A in 0.1M-KH₂PO₄.

The molecular weight of the polysaccharide calculated by means of the Svedberg equation (Svedberg & Pedersen, 1940) from these values was 32 000.

The number-average molecular weight of the polysaccharide was determined by labelling the reducing end of the polysaccharide with Na¹⁴CN by the method described by Isbell (1951) and Moyer & Isbell (1958). The dry radioactive samples were counted in a proportional internal gas-flow counter (Nuclear-Chicago Corp., U.S.A.). The reactions were carried out in constricted test tubes (9.5 cm. $\times$ 8.8 mm.). Controls containing 20 μ l. of glucose solution (0.02 m-mole), 10 μ l. of NH₄Cl buffer (0.6M) and 20 μ l. of Na¹⁴CN (0.6 mc) and a drop of sulphur-free toluene were used. The blank contained 20 μ l. of water instead of the sugar. The polysaccharide, 4.36 or 5.00 mg., replaced the glucose in the experimental tubes. All the experiments were carried out simultaneously on either three or five samples. The results are shown in Table 2. A value of 35 000 was obtained for the number-average molecular weight.

Viscosity measurements. The results are shown in Fig. 8. The intrinsic viscosity $[\eta]$ was 0.35 dl./g. The viscosity increment v , calculated from the intrinsic viscosity and the partial specific volume, was 36.

An experiment showed that the viscosity of the polysaccharide measured at varying pressures was independent of the rate of shear. Varying pressures between 30 and 70 cm. water were applied for all concentrations. Thus the flow of the solution was strictly Newtonian (Alexander & Johnson, 1949).

The axial ratios obtained from the viscosity increment with Simha's equation (Simha, 1940; Mehl, Oncley & Simha, 1940), assuming that the molecule was an unhydrated ellipsoid of revolution, were 20 for a prolate ellipsoid and 60 for an oblate ellipsoid.

Table 2. Number-average molecular weight of hemicellulose A

Labelling of reducing end of the polysaccharide was done with sodium[¹⁴C]cyanide.

Net counts/min. for D-glucose control (0.02 m-mole) (three samples)

A	B	C
13 977	13 977	14 244
13 977	13 774	13 977
14 286	13 946	13 972

Average 13 977

Net counts/min. for sucrose control (0.02 m-mole) (three samples)

A	B	C
26	26	15
26	11	26
10	16	10

Net counts/min. for hemicellulose A (five samples)

4.360 mg.	5.007 mg.
76 923	100 000
76 923	99 980
77 770	100 000
77 280	100 000
77 459	100 000

Average 77 272 Average 99 987

Number-average molecular weight 3.5×10^4 3.9×10^4

Fig. 8. Viscosity of hemicellulose A in aqueous solution at 23.5°.

Frictional coefficient (f). This was calculated from the constants S_{20} , D_{20} and $\bar{V}$, with the equation of Oncley (1941) and Perrin (1936). The value of 1.78 obtained, assuming that the polysaccharide was a rigid unhydrated ellipsoid of revolution, corresponded to axial ratios of 15 for a prolate ellipsoid and 20 for an oblate ellipsoid (Svedberg & Pedersen, 1940).

By the application of the equations of Scheraga & Mandelkern (1953) to the physicochemical data for hemicellulose A, a value of 2.71×10^6 was obtained for β (β is defined as the parameter which is a function of the axial ratio of an ellipsoid of revolution). This value of β corresponded to a prolate ellipsoid with an axial ratio of 25.

Composition of hemicellulose A

The polysaccharide (20 mg.) was hydrolysed and the hydrolysate examined chromatographically. The molar ratios of the sugars present are shown in Table 3.

Identification of sugars. A mixture of monosaccharides (200 mg.) was obtained by elution with water of a partial acid hydrolysate (1.24 g.) from a charcoal-Celite column. The mixture was separated on a cellulose column. The sugars were recrystallized from methanol and characterized as follows: galactose, by preparation of the diethyl mercaptal derivative (m.p. and mixed m.p. 140–141°) (Wolfson, 1930); glucose, by preparation of the *N*-*p*-nitrophenyl-D-glucopyranosylamine dihydrate (m.p. and mixed m.p. 182–184°) (Barker, Foster, Siddiqui & Stacey, 1958); mannose, by preparation of the phenylhydrazine (m.p. and mixed m.p. 191–192°) (Hirst, Jones & Woods, 1947); arabinose, by preparation of the benzoylhydrazone (m.p. and mixed m.p. 199–201°) (Hirst *et al.* 1947); xylose, by preparation of the dibenzylidene dimethylacetal derivative (m.p. and mixed m.p. 211°) (Breedy & Jones, 1945); rhamnose, by preparation of the benzoylhydrazone (m.p. and mixed m.p. 179–180°) (Hirst *et al.* 1947).

Configuration of galactose and rhamnose. This was investigated by selective fermentation of galactose with a yeast strain, *Saccharomyces delbreuckii* var. *mongoliensis* (kindly supplied by Dr R. Davies). The polysaccharide (25 mg.) and snail galactan (25 mg.) [known to contain both D- and L-galactose (Baldwin & Bell, 1941)] were hydrolysed and the neutralized hydrolysates each made up to 5 ml. D-Galactose (25 mg.) was also dissolved in 5 ml. of water. To each solution the yeast suspension was added and fermentation carried out at room temperature for 3 hr. The yeast suspension was centrifuged off and the clear supernatant in each case concentrated and examined chromatographically. Only the snail galactan still showed the presence of galactose, and further treatment with fresh yeast suspension did not remove any more galactose from it. The residual galactose of the snail galactan was recrystallized from aq. 70% ethanol; $[\alpha]_D^{20} - 80.1^\circ$. The galactose in the hemicellulose, which was all removed by the yeast fermentation, was therefore present as the D-galactose.

The hemicellulose (250 mg.) was hydrolysed, and all the galactose removed by fermentation with the yeast suspension. The galactose-free solution was applied to a cellulose column and the rhamnose eluted and recrystallized from methanol; $[\alpha]_D^{20} + 9.0^\circ$ (c 0.90 in water; l, 1). The rhamnose was therefore present as the L-rhamnose.

Enzymic hydrolysis of hemicellulose A

Salivary amylase. The polysaccharide (10 mg. in 2 ml. of water) was incubated with 0.5 ml. of salivary amylase at 37° for 48 hr. under toluene. At the end of the incubation the enzyme was inactivated by heating at 60° for 5 min. and the solution dialysed against 200 ml. of water. The solution outside the bag was centrifuged, concentrated and examined chromatographically. No breakdown product was found. The material inside the bag when hydrolysed contained all the sugars of the original hemicellulose.

Cellulase. The hemicellulose (25 mg.) was dissolved in acetate buffer (5 ml.; pH 5.6) and incubated with cellulase (1 ml.) at 37° under toluene for 48 hr. Control experiments were carried out concurrently with enzyme in the absence of polysaccharide and with polysaccharide in the absence of enzyme. The enzyme was inactivated by heating at 75° for 30 min. and the solution dialysed against 1 l. of distilled water for 8 hr. Sodium ions were removed from the solution outside the bag by passing it through a bed of Amberlite resin IR-120 (H), and the products were examined chromatographically. Glucose, a faint trace of galactose and two oligosaccharides were found. The oligosaccharides were eluted from the paper and hydrolysed. They contained galactose, mannose and rhamnose, with traces of arabinose and xylose. The enzyme-resistant material, inside the bag, was reincubated with fresh enzyme for 48 hr. and again for 84 hr. until an enzyme-resistant core was obtained. This, on hydrolysis, contained the sugars of the original polymer. The control solutions contained no breakdown products.

β -Galactosidase. The polysaccharide (13 mg.) was dissolved in KH_2PO_4 buffer (3 ml.; pH 7.0) and 0.005 μM - MgCl_2 . The solution was incubated with 0.5 ml. of enzyme under toluene. Control experiments were carried out concurrently with enzyme in the absence of polysaccharide, and polysaccharide in the absence of enzyme. The enzyme was inactivated by heating at 60° for 5 min., and the solution dialysed against 500 ml. of distilled water for 8 hr. Salts were removed from the solution outside the bag, after concentration by evaporation, by passing it successively through Amberlite resins IR-120 (H) and IR-4B (OH). The products of breakdown were found to be galactose, very faint traces of arabinose and mannose and two oligosaccharides. The oligosaccharides, on hydrolysis, contained galactose, mannose, arabinose and rhamnose. After two further incubations of the enzyme-resistant material with fresh enzyme for 40 hr. each, an enzyme-resistant core was obtained which contained all the sugars of the original polymer. The controls showed no breakdown products.

Periodate oxidation of hemicellulose A

Determination of formic acid released. The polysaccharide (47 mg.) was dissolved in a solution (85 ml.) containing KCl (4 g.) and 5 ml. of 0.3M-sodium metaperiodate was added to it. A control solution without polysaccharides was investigated simultaneously. The solutions were kept in the dark at room temperature and continuously agitated

Table 3. Composition of hemicellulose A

Sugars	Molar ratios of sugars (35 experiments)	
	Method of Timell, Glaudemans & Currie (1956)	Method of Wilson (1959)
Galactose	6.8	7.2
Glucose	1.0	1.0
Mannose	2.5	2.0
Arabinose	3.0	3.0
Xylose	3.0	3.0
Rhamnose	3.0	5.0

on a Microid flask shaker (Griffin and George Ltd.). Samples (10 ml.) were taken at intervals, treated with ethylene glycol (0.5 ml.) and the formic acid was titrated against NaOH (0.01N), methyl red being used as indicator (Halsall, Hirst & Jones, 1947; Hirst, Jones & Roudier, 1948). The numbers of moles of formic acid released per anhydrohexose unit at times 12, 24, 72 and 118 hr. were 0.21, 0.25, 0.31 and 0.34 respectively. Thereafter, there was no further change up to 480 hr.

Periodate uptake and isolation of the oxopolysaccharide. The polysaccharide (100.5 mg.) was dissolved in water (110 ml.) and oxidized with 0.3M-sodium metaperiodate (10 ml.) in the dark, with constant agitation. The periodate used was determined at intervals by the method of Fleury & Lange (1933) and Halsall *et al.* (1947). The results are shown in Fig. 9.

The periodate oxidation was continued for 30 days, after which ethylene glycol (1 ml.) was added to stop the reaction. Excess of periodate was precipitated with barium acetate (0.05M) and the supernatant was dialysed for 48 hr. against running tap water and for 24 hr. against distilled water. The dialysed solution was concentrated and precipitated in ethanol (125 ml.). Yield of oxopolysaccharide, 16 mg. (16%). The oxidized material (6 mg.) was hydrolysed and the sugars were quantitatively estimated: galactose (4.0 moles), rhamnose (2.0 moles), arabinose (1.5 moles), xylose (1.5 moles), glucose (1.0 mole) and mannose (0 mole).

The remaining oxopolysaccharide (10 mg.) was reduced with potassium borohydride (10 mg.) and the polyalcohol obtained hydrolysed and examined chromatographically (Abdel-Akher, Hamilton, Montgomery & Smith, 1952). Galactose, glucose, arabinose, xylose and rhamnose were found, together with glycerol and an unidentified alcohol.

Hydrolysis of hemicellulose A

Mild acid hydrolysis. The polysaccharide (2.5 mg.) was hydrolysed with 1M-N-H₂SO₄ at 100° for 1 hr. and examined chromatographically. No sugar spots were obtained (Jevons, 1958; Winzler, 1955).

The polysaccharide (28.0 mg.) was hydrolysed with 0.01N-H₂SO₄ at 100° for 14 hr. (Haworth, 1932). The neutralized solution was dialysed against 1 l. of distilled

water for 8 hr. The solution outside the bag was concentrated and examined chromatographically. Traces of galactose, rhamnose, mannose, arabinose and xylose were found.

Partial acid hydrolysis. The polysaccharide (805 mg.) was partially hydrolysed, neutralized, concentrated and applied to a charcoal-Celite column. The oligosaccharides eluted by all ethanol concentrations proved to be complex mixtures of di- and tri-saccharides. Some of the mixtures were composed of galactose and rhamnose only and the others contained galactose, mannose and rhamnose or galactose, mannose, arabinose and rhamnose. The similar nature of these oligosaccharides did not permit purification on a cellulose column, by paper chromatography in various solvent systems, by paper electrophoresis at low or high voltage, or by preparative glass-powder column electrophoresis.

A mixture of sugars (galactose, 350 mg.; rhamnose, 250 mg.; arabinose, 150 mg.; xylose, 150 mg.; mannose, 100 mg.; glucose, 50 mg.) was partially hydrolysed under the same conditions as in the above-mentioned experiment and applied to the charcoal-Celite column. No evidence for the formation of oligosaccharides was obtained.

Acetolysis of hemicellulose A

The polysaccharide (1 g.) was added in small quantities to a mixture of acetic anhydride (8 ml.), acetic acid (8 ml.) and conc. H₂SO₄ (0.4 ml.) and kept at 0°. After 14 hr. the mixture was incubated successively at 37° for 9 days, and at 25° for 14 days. The insoluble residue (432 mg.) was removed and the clear supernatant was added to ice-cold water (150 ml.) and the solution neutralized with NaHCO₃ and extracted with CHCl₃. The aqueous layer was evaporated to dryness and the crude solid dried and extracted repeatedly with ethanol-ether-benzene (1:3:1, by vol.). The solution was concentrated and precipitated in light petroleum (b.p. 60–80°), when white needle-like crystals were obtained, and these were recrystallized from ethanol-ether (1:1). The oligosaccharide acetate was deacetylated with potassium ethoxide in ethanol and the free oligosaccharide recrystallized from methanol.

The oligosaccharide moved as one spot, electrophoretically and chromatographically. It contained galactose, arabinose and rhamnose in the ratio 1:1:1. The sugar residues were stable to mild acid hydrolysis (0.01N-H₂SO₄ at room temp. for 2 hr., followed by heating at 100° for 2 hr.).

The molecular weight of the oligosaccharide found by measurement of reducing power (Somogyi, 1945; Isbell & Snyder, 1953) was 528.

The oligosaccharide (10 mg.) was dissolved in 5 ml. of water and an equal quantity of potassium borohydride added; the mixture was kept at room temperature for 30 hr. Acetic acid was added drop by drop to decompose excess of borohydride and the solution was passed through a bed of Amberlite resin IR-120 (H). The solution was concentrated and evaporated four times with methanol and evaporated to a syrup (9.03 mg.). The reduced oligosaccharide (4 mg.) was hydrolysed and gave rhamnitol, galactose and arabinose. Oxidation with sodium metaperiodate (0.15M) for 100 hr. destroyed all the sugar residues in the oligosaccharide.

The acetolysis procedure carried out on a mixture of free sugars failed to yield any oligosaccharides.

Fig. 9. Oxidation of hemicellulose A (100.5 mg.) by sodium metaperiodate.

Acetylation and methylation of hemicellulose A

Acetylation. The polysaccharide (700 mg.) was dispersed in pyridine (80 ml.) and acetic anhydride (70 ml.), added in small quantities, with mechanical stirring. The mixture was acetylated at 37° for 20 days. At the end of this time the insoluble material (190 mg.) was removed by filtration, and the clear solution extracted with CHCl_3 . The CHCl_3 layer was dried over anhydrous Na_2SO_4 and precipitated in light petroleum (b.p. 60–80°). The brown precipitate obtained was collected and dried. Yield, 405 mg.; acetyl content, 39.3%.

Methylation. The acetylated polysaccharide (400 mg.) was dissolved in acetone and methylated with 30% NaOH and dimethyl sulphate at 37° (Haworth & Percival, 1932). After the first nine methylations, the solution was dialysed, concentrated and remethylated nine times again. The final solution was dialysed, concentrated and extracted with CHCl_3 . The CHCl_3 layer was dried over anhydrous Na_2SO_4 and precipitated in light petroleum (60–80°). A brown product was obtained (yield, 57.0 mg.; OMe, 43.7%).

Hydrolysis of the methylated hemicellulose and separation of cleavage products

The methylated polysaccharide (8.2 mg.) was refluxed for 15 hr. with 5% (w/v) HCl (1 ml.) and acetic acid (1 ml.). After neutralization with BaCO_3 , the filtrate was evaporated to dryness and the solid residue extracted with CHCl_3 . The CHCl_3 layer was concentrated to a syrup and examined chromatographically. Five spots were detected: (a) a spot identical with a 2,3,4,6-tetra-*O*-methyl-D-galactose marker; (b) a spot identical with 2,3,6-tri-*O*-methyl-D-galactose marker; (c) a spot identical with a dimethylgalactose marker; (d) a spot identical with 2,3,4,6-tetra-*O*-methyl-D-mannose marker; (e) an unidentified spot.

Reduction of hemicellulose A with potassium borohydride

The polysaccharide (10 mg.) in aqueous solution was treated with potassium borohydride (10 mg.) at room temperature for 72 hr., and the reduced polysaccharide was hydrolysed and examined chromatographically. In addition to the free reducing sugars of the polysaccharide, a small amount of rhamnitol was found.

Properties of *Chlorella* starch

Characterization of glucose. After complete hydrolysis of the starch, the glucose obtained was characterized by preparation of the *N*-*p*-nitrophenylglucopyranosylamine dihydrate (m.p. 182–184°).

Salivary-amylase action. The polysaccharide (15 mg.) was dissolved in 3 ml. of water and salivary amylase (1 ml.) added. Incubation at 37°, under toluene, was carried out for 18 hr. A solution containing soluble starch (1%) was used as a control. After incubation, chromatographic investigation of the products of breakdown showed the presence of glucose and maltose only.

Periodate oxidation of *Chlorella* starch. The polysaccharide (25 mg.) was dissolved in 45 ml. of water and 5 ml. of 0.3M-sodium metaperiodate and 4 g. of KCl were added. Oxidation was carried out at room temperature, in the dark, with shaking. A solution containing no polysaccharide was also investigated. Samples (10 ml.) were analysed for

formic acid at intervals. The number of moles of formic acid released per anhydrohexose unit at times 24 and 72 hr. were 0.07 and 0.12 respectively. Thereafter there was no further change up to 216 hr. The result indicated an apparent chain length (glucose residues) of 9.

Periodate uptake was measured by dissolving the polysaccharide (16 mg.) in 55 ml. of water and adding 5 ml. of 0.3M-sodium metaperiodate. A solution containing no polysaccharide was also investigated. The reduction of periodate was measured at intervals, with 2 ml. portions. After 12 hr. 0.77 mole of periodate was consumed per anhydrohexose unit. This value was unchanged after 536 hr.

After oxidation for 30 days the oxopolysaccharide was isolated: yield, 1 mg. (6%). Glucose was detectable in acid hydrolysates of this material. The experiment was repeated after an oxidation of 41 days, when glucose could still be detected in the acid hydrolysate of the oxopolysaccharide.

Fractionation of *Chlorella* starch. The polysaccharide (30 mg.) was dissolved, with mechanical stirring, in 40 ml. of boiling water. After boiling for 20 min. solid NaCl was added and the solution was rapidly cooled to room temperature. Powdered thymol (14 mg.) was stirred into the solution, which was then stirred at room temperature for 2 hr. (Haworth, 1946). The thymol-amylose complex formed and the mixture was left at 4° for 87 hr. The complex was collected, washed rapidly and successively with water saturated with thymol, ethanol and ether, and then dried to form a fine white powder; yield, 2 mg. (7%). It gave an intense-blue colour with iodine.

The supernatant solution containing the amylopectin was added to ethanol and the white product obtained was washed with ethanol and acetone and dried. Yield, 24 mg. (80%).

DISCUSSION

For the results of structural analysis of polysaccharides to be of significance, some criteria of purity and homogeneity must be applied to the polymers studied. There has been no report on the homogeneity of polysaccharides investigated from green algae. In this work, we have described the isolation of alkali-soluble polysaccharides from the green alga *Chlorella pyrenoidosa* and established some definite criteria of purity for the components fractionated.

The electrophoretic mobilities of hemicellulose B and *Chlorella* starch are the same (Fig. 5a, b, c), and this explains why the starch isolated after electrophoresis on a glass column appeared homogeneous by boundary electrophoresis. This result indicates the need for caution in the interpretation of electrophoretic results and the necessity for a number of diverse fractionation procedures to be applied before any conclusions about homogeneity of materials are drawn.

Hemicellulose A. The variation of sedimentation coefficient with polysaccharide concentration is evident. This indicated that the molecule was asymmetrical and this has been confirmed by the

results of viscosity measurement. The calculations of the axial ratios and the frictional coefficient indicated that the molecule was a prolate ellipsoid of revolution with an axial ratio of 15.

The molecular weight is comparatively large for hemicelluloses. The value for the molecular weight determined by the ultracentrifuge (weight average), and the value determined from the radioactive-cyanide-labelling experiment (number average) are almost equal. Agreement of this nature is to be expected only if the polysaccharide investigated is homogeneous and relatively little polydisperse. That this was true for hemicellulose A was again indicated by the sharp single peaks which were obtained when the material was investigated in the ultracentrifuge and the Tiselius electrophoresis apparatus.

The liberation of glucose by cellulase digestion of hemicellulose A suggested that some of the glucose residues were β -linked. Similarly, the hydrolysis of some of the galactose residues by β -galactosidase indicated that some of these units in the original polymer were β -linked (Monod, Cohen-Bazire & Cohn, 1951; Wallenfels & Malhotra, 1960). Hemicellulose A had, as would be expected for a β -linked polymer, a low specific optical rotation.

The sugars in hemicellulose A are present in the pyranose form since weak acid hydrolysis failed to liberate any of the residues. The results of partial acid hydrolysis indicated that continuous units of one sugar, either as a chain or as separate polymers in a mixture of polysaccharides, are not present, since no oligosaccharide or residual material containing units of one sugar were isolated.

Further evidence that galactose, arabinose and rhamnose occurred in one polysaccharide was obtained by the isolation and characterization of a trisaccharide containing these three sugars. This was obtained from an acetolysis mixture of hemicellulose A. This trisaccharide had rhamnose at its reducing end and since periodate oxidation cleaved all the sugar units these were joined by linkages other than 1 $\rightarrow$ 3.

The results from experiments with periodate oxidation of hemicellulose A indicated that the molecule was complex and highly branched. The high periodate uptake (Fig. 9), especially the high initial values, showed that at least 2 moles of periodate/anhydrohexose unit were consumed. This is possible only if 1 $\rightarrow$ 6 linkages are present. The high yield of formic acid from the periodate oxidation also suggested the presence of this linkage. The oxopolysaccharide still contained unchanged sugar residues and these resistant units were therefore branched or linked 1 $\rightarrow$ 3 in the original polysaccharide. Mannose was absent from the oxopolysaccharide and thus this sugar occupied terminal positions or was unbranched.

The polysaccharide molecule is large and difficult to acetylate and methylate. The presence of 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,3,4,6-tetra-*O*-methyl-D-mannose in the methylated polysaccharide showed that some of the galactopyranose and mannopyranose residues functioned as end units, whereas the presence of a dimethyl-galactose suggested that branching occurred on this residue. These results confirmed some of the findings of the periodate experiments.

Chlorella starch. This polysaccharide seemed to be closely associated with hemicellulose and in some cases it was impossible to remove starch by digestion of the initial crude mixture of the alkali-soluble polysaccharides with salivary amylase. However, when the starch was separated from the hemicellulose A on the preparative electrophoresis column it was easily digested by the amylase, although it was still mixed with hemicellulose B. This latter polysaccharide was removed by fractionation with cetyltrimethylammonium bromide.

The starch could be separated into amylose and amylopectin and these gave characteristic colours with iodine.

The results of the periodate studies on this material indicated that the average chain length was short, being about nine glucose residues. Prolonged oxidation of the polysaccharide failed to remove completely all the glucose residue and it is possible that linkages other than 1 $\rightarrow$ 4 and 1 $\rightarrow$ 6 were present.

SUMMARY

1. Three polysaccharides, hemicellulose A, hemicellulose B and starch, present in the ratio of 9:2:1 respectively, have been isolated and purified from the alkali-soluble polysaccharides of *Chlorella pyrenoidosa*.

2. Hemicellulose A has been shown to be homogeneous by several different criteria and its molecular weight, determined by the ultracentrifuge and end-group assay with sodium[^{14}C]cyanide is 32 000–35 000.

3. The physicochemical data for hemicellulose A have shown that the molecule is a prolate ellipsoid of revolution with an axial ratio of 15.

4. The composition of hemicellulose A was shown to be galactose (7.0 moles), glucose (1.0 mole), mannose (2.0 moles), arabinose (3.0 moles), xylose (3.0 moles) and rhamnose (5.0 moles). Yeast fermentation of the hydrolysate showed that D-galactose and L-rhamnose were present.

5. The optical rotation and the enzymic hydrolysis of hemicellulose A by cellulose and β -galactosidase indicated the presence of β -linkages.

6. A trisaccharide containing galactose, arabinose and rhamnose was isolated from the acetolysis mixture of hemicellulose A.

7. Periodate oxidation and methylation experiments showed that the molecule of hemicellulose A was branched and that 1→6 linkages were present. Branching occurred on some of the galactose residue and mannose and galactose occurred as terminal units.

8. Hemicellulose B was found to contain glucose, galactose, mannose, arabinose, xylose, rhamnose and an unidentified sugar.

9. *Chlorella* starch was composed of amylose (7 %) and amylopectin. The average chain length was nine glucose units, and links other than 1→4 and 1→6 were possibly present.

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